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Lentiviral Vectors

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## **Introduction**

Tumor progression induces the growth of endothelial cells by releasing angiogenic factors. This is accompanied by down-regulation of local tissue inhibitors of endothelial cell proliferation such as angiostatin and endostatin. Both proteins target normal endothelial cells and effectively regress large tumors in animals. However, animal studies demonstrate that an effective treatment requires long-term administration of angiogenesis inhibitors. Thus, delivery of angiogenesis inhibitor genes to tumor sites should increase local concentration of these proteins, leading to the retardation of tumor progression and metastasis. We propose to use HIV vectors to deliver the endostatin and angiostatin genes into human prostate cancer cell lines in culture. The effect of these two proteins will be evaluated by tumor formation and metastasis in nude mice grafted with the transduced cells. During the past fiscal year, we have established stable human prostate PC3 cell lines expressing either endostatin, angiostatin or both angiogenesis inhibitors together. The expression of these proteins had no effect on the growth rate of PC3 cells. However, culture supernatant harvested from these cell lines exhibited growth inhibition of primary human endothelial cells in culture. We used two different assays to detect potential contamination of replication competent lentivirus (RCL) in these established cell lines and failed to detect the presence RCL. We have now implanted these cells onto nude mice and are in the process of evaluating the effect of the angiogenesis inhibitor on tumor growth *in vivo*.

## Body

During this fiscal year, we have established stable cell clones derived from human prostate cancer cell line, PC3, expressing angiogenesis inhibitors. These transduced cells will then be tested for their tumorigenicity *in vivo*. PC3 cells were transduced with either HIV7/GFP containing only the GFP gene, HIV7/endo containing the endostatin cDNA, HIV7/angio containing the angiostatin cDNA or both HIV7/endo and HIV7/angio at high multiplicity of infection (MOI). Since both HIV7/endo and HIV7/angio also carry the GFP gene, the efficiency of PC3 transduction can be determined by the FACS analysis of GFP+ cells. As shown in Fig. 1, at an MOI of 5, approximately 20% of the

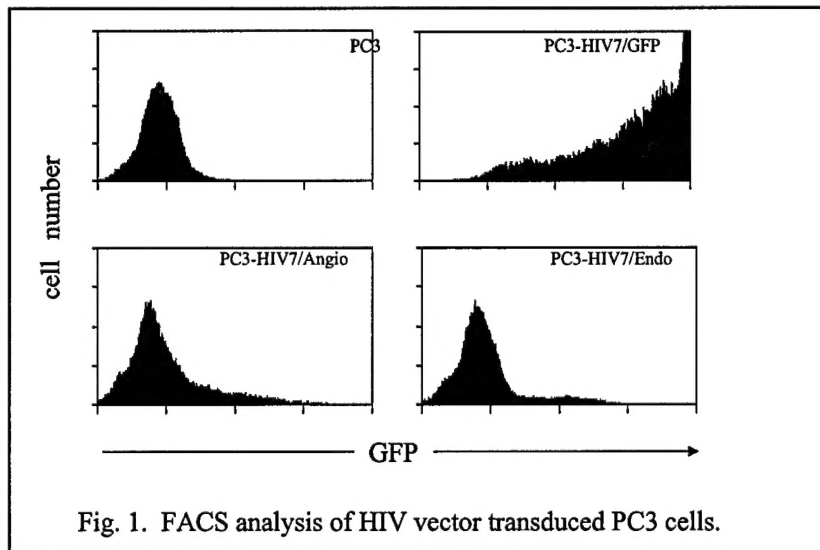


Fig. 1. FACS analysis of HIV vector transduced PC3 cells.

PC3 cells became GFP+. In contrast, more than 90% of HIV7/GFP transduced cells became GFP+ with the same MOI. To increase the fraction of cells expressing the angiogenesis

inhibitor, PC3 cells were repeatedly transduced with the HIV vectors at the MOI of 5. As shown in Table 1, the fraction of GFP+ cells increased to 84% and 95% when transduced

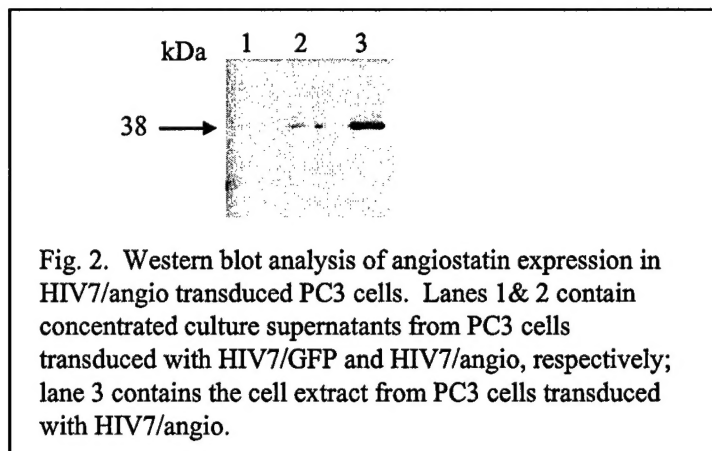
Table 1. Percentage of GFP+ PC3 cells transduced with different HIV vectors.

mock	HIV7/ GFP	HIV7/ endo	HIV7/ angio	HIV7/endo + HIV7/angio
0%	95%	84%	95%	80%

with HIV7/endo and HIV7/angio, respectively. To determine the combined effect of endostatin and angiostatin, we mixed equivalent fractions of HIV7/endo and HIV7/angio

transduced PC3 cells. The GFP+ fraction in this population was determined to be 80% (HIV7/endo + HIV7/angio in Table 1). Since endostatin was tagged with the influenza HA peptide [1], to determine whether angiostatin was expressed and at what level, the culture supernatant or cell extract from PC3 cells transduced with either HIV7/GFP or

HIV7/angio were prepared and subjected to Western blot analysis using a HA-specific antibody. As shown in Fig. 2, angiostatin expression was readily detectable in the culture

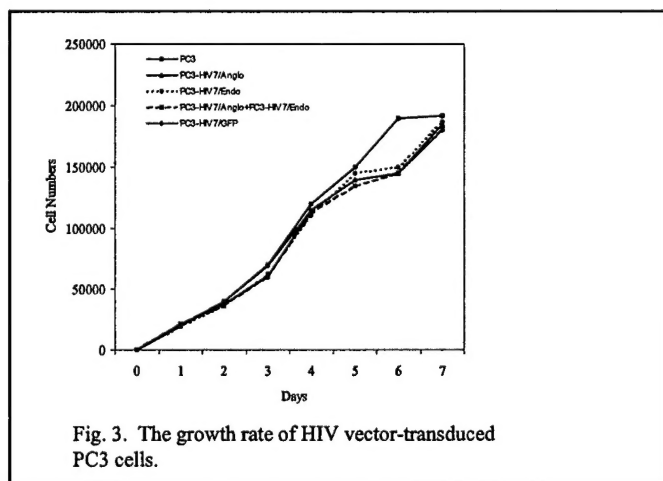


supernatant and the cell extract derived from PC3 cells transduced with HIV7/angio but not in PC3 cells

transduced with HIV7/GFP.

Since the antibody specific for endostatin is not currently available, we were not able to perform a similar experiment

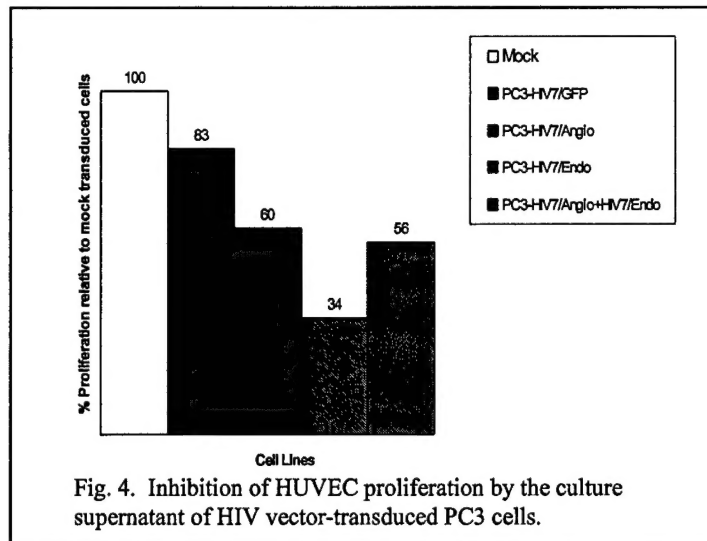
to evaluate endostatin expression. To determine whether expression of the angiogenesis inhibitor affects the proliferation of PC3 cells, the growth rate of these cell lines were determined. As shown in Fig. 3, no distinct difference in the growth rate of these cells



could be detected. To determine whether the angiogenesis inhibitors produced from these cell lines were capable of blocking the proliferation of normal endothelial cells, the culture supernatant from each cell lines was harvested and concentrated with Centricon [2].

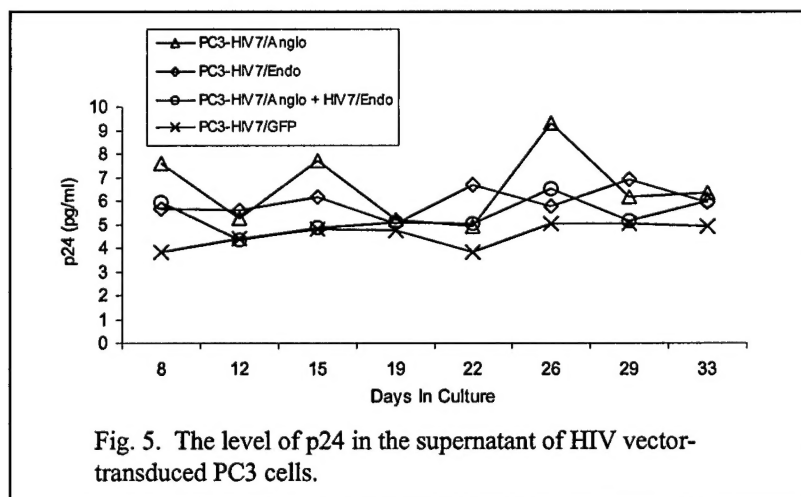
The concentrated supernatant was applied to primary human umbilical vein endothelial cells (HUVEC) and cell proliferation was monitored by the MTT assay. We have shown in the last progress report that direct transduction of HUVECs with HIV7/endo or HIV7/angio led to a decrease in the cell proliferation rate in culture. As shown in Fig. 4, the culture supernatants harvested from PC3 cells transduced with HIV7/endo, HIV7/angio and HIV7/endo plus HIV7/angio inhibited HUVEC proliferation whereas the supernatants harvested from PC3 cells transduced with HIV7/GFP had little effect on HUVEC proliferation. A combination of endostatin and angiostatin, however, did not

have any additive effect on the inhibition of HUVEC proliferation. This experimental



approach mimics the *in vivo* gene therapy conditions where vector-transduced cancer cells express and secrete the angiogenesis inhibitor to block normal endothelial cell proliferation. Before implanted the transduced cells into nude mice to assess the effect of angiogenesis

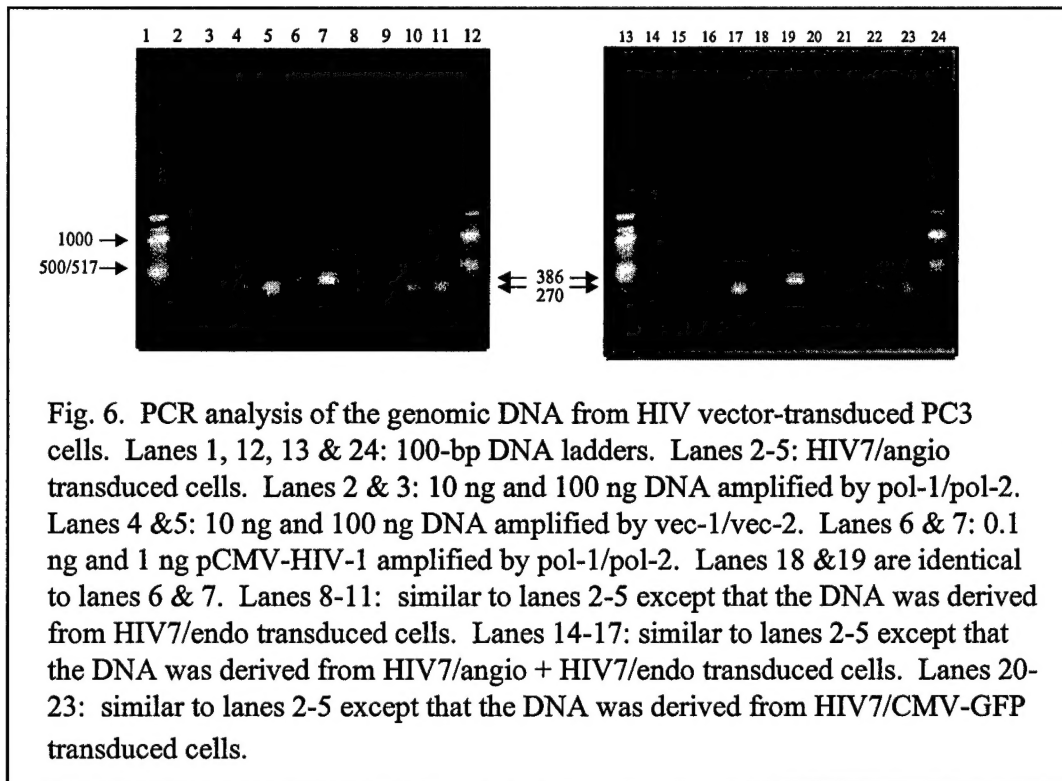
inhibitors on tumor growth *in vivo*, we used two assays to detect the potential contamination of replication competent lentivirus (RCL) in the established PC3 lines. In the first assay, the transduced PC3 cells were cultured continuously for over a month and the culture supernatant was collected during this period and the p24 level was then determined using a commercially available Elisa kit. As shown in Fig. 5, no significant



level of p24 could be detected in any of the transduced PC3 cells at the end of this culture period. The values of p24 fell below the detection limit of 7 pg/ml. In the second assay, genomic DNA from

the transduced cells was prepared at the end of the culture period and subjected to DNA PCR analysis to detect potential RCL. Two pairs of PCR primers were used: pol-1/pol-2 amplify a 386-bp fragment in the reverse transcriptase (RT) gene. RCL is expected to contain this gene in order to replicate and spread. As a control, vec-1/vec-2 amplify a 270-bp fragment presence in the 5' untranslated region of HIV-1. Both RCL and the HIV

vectors used in this study are expected to contain this sequence. As shown in Fig. 6, the 386-bp fragment could only be detected from PCR amplification of pCMV-HIV-1 which contains the RT gene (lanes 6, 7, 18 & 19). None of DNA from the transduced cells contained this gene. In contrast, the 270-bp fragment was readily detectable in the DNA samples isolated from the transduced cells. We concluded that the PC3 cell lines established were free of RCL contamination. To assay the effect of angiogenesis inhibitor expression *in vivo*, we first determined that approximately  $2 \times 10^6$  PC3 cells were required to induce tumor in nude mice within 10 days with subcutaneous implantation. Five groups of nude mice with five mice in each group were then implanted with  $2 \times 10^6$  PC3, PC3/HIV7/GFP, PC3/HIV7/endo, PC3/HIV7/angio and PC3/HIV7/endo plus HIV7/angio, respectively. This is ongoing and tumor growth *in vivo* will be followed by measuring the tumor size.



### **Key Research Accomplishments**

1. Establish stable PC3 cell lines stably expressing endostatin, angiostatin, or both.
2. Demonstrate the inhibition of primary endothelial cell proliferation with the angiogenesis inhibitors produced from the stable PC3 cell lines
3. Demonstrate the absence of RCL in the established cell lines and proceed with nude mice implantation of these cell lines to evaluate tumor formation *in vivo*

### **Reportable Outcomes**

1. Establish PC3 cell lines stably expressing endostatin and angiostatin. These cell lines can be used to evaluate prostate tumor progression *in vivo*.
2. Claudia Kowolik, a Postdoctoral Fellow, received training in HIV vector production, RCL detection and tumor cell implantation onto nude mice.

### **Conclusion**

1. Stable human PC3 prostate cancer cell lines expressing endostatin and angiostatin were established.
2. Expression of these angiogenesis inhibitors had no effect on PC3 cell proliferation in culture.
3. The angiogenesis inhibitors produced from PC3 cells, however, inhibited the proliferation of primary human endothelial cells in culture.
4. No RCL could be detected from these stable PC cell lines. These cell lines therefore can be used to implant onto nude mice.

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## **Appendices**

### **1. Curriculum Vitae**

## **CURRICULUM VITAE**

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1972 - 1976	B.S. in Biology, Fu Jen Catholic University, Taipei, Taiwan
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- |              |  |
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| 1976 - 1977  | Teaching Assistant for laboratory courses in Biochemistry and Microbiology,<br>Department of Biology, Fu Jen Catholic University, Taipei, Taiwan |
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| 1994 - 1996  | Instructor for the course of Molecular and Cellular Biology in the Graduate Program, City of Hope, Duarte, California                            |
| 2001-present | Instructor for Breast Cancer Biology in the Graduate Program, City of Hope, Duarte, California   |

**Scientific Committees:**

- Ad hoc Reviewer for NIDDK study section (1991)
- Ad hoc Reviewer for Wellcome Trust Foundation (1991)
- Ad hoc Reviewer for NIDDK study section (1992)
- Ad hoc Reviewer for NIDDK study section (1994)
- Reviewer for NIH SBIR Special Study Section (2000- 2001)
- Ad hoc Reviewer for NIH Recombinant DNA Advisory Committee (2001)

**Grant Support:**

Project Title: Hematopoietic cell transplantation for hematologic malignancy		
P01 CA30206	NIH/NCI	4/1/00 - 3/31/05
Role: Co-PI (PI: S. Forman)	20 % effort	

Project Title: Transduction of ribozymes into CD34+ stem cell in AIDS

P01 A146030

NIH

9/1/99-8/31/03

Role: Project Leader (PI: J. Zaia) 30% effort

Project Title: Anti-angiogenic gene therapy of prostate cancer with lentiviral vectors

New Investigator Award Prostate Cancer Research Program, US Army

03/01/01-02/28/04

Role: PI 10% effort

**Patent granted**

Patent No. 6,133,027

Title: Inducible expression system

Inventors: **Yee; Jiing-Kuan**; Friedmann; Theodore; Chen; Shin-Tai

Assignee: City of Hope (Duarte, CA); The Regents of the University of California (Oakland, CA)

Patent No. 5,817,491

Title: VSV G pseudotyped retroviral vectors

Inventors: **Yee; Jiing-Kuan**; Emi; Nobuhiko; Friedmann; Theodore; Jolly; Douglas J.; Barber; Jack R.

Assignee: The Regents of the University of California (Oakland, CA); Chiron Viagene, Inc. (Emeryville, CA)

Patent No. 5,739,018

Title: Packaging cell lines for pseudotyped retroviral vectors

Inventors: Miyanochara; Atsushi; **Yee; Jiing-Kuan**; Chen; Shin-Tai; Prussak; Charles Edward; Friedmann; Theodore

Assignee: The Regents of the University of California (Oakland, CA); City of Hope (Duarte, CA)

Patent No. 5,670,354

Title: Use of VSV-G pseudotyped vectors for transfer of genes into embryos

Inventors: Burns; Jane C.; **Yee; Jiing-Kuan**; Friedmann; Theodore

Assignee: The Regents of the University of California (Oakland, CA)

Patent No. 5,512,421

Title: Generation, concentration and efficient transfer of VSV-G pseudotyped retroviral vectors

Inventors: Burns; Jane C.; **Yee; Jiing-Kuan**; Friedmann; Theodore

Assignee: The Regents of the University of California (Oakland, CA)

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